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Ameson Michaelis (Microsporida) in the Blue Crab, Callinectes Sapidus: Altered Host Cell and Isolated Parasite Metabolism.

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AMESON MICHAELIS (MICROSPORIDA) IN THE BLUE CRAB, CALLINECTES SAPIDUS: ALTERED HOST CELL AND ISOLATED PARASITE METABOLISM

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Ameson michaelis (Microsporida)

in the blue crab, *Callinectes sapidus*:

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ABSTRACT

The Microsporida are a large group of highly specialized obligate intracellular protozoan parasites. Despite their wide distribution in nature and their devastating effect on a variety of animal populations, relatively little is known about the physiological characteristics of these parasites or of their interactions with host cell elements. In the present study, we have investigated the biochemical and physiological activities of Ameson michaelis (Microsporida) during growth within the blue crab, Callinectes sapidus. The ultimate objectives have been to identify some of the mechanisms by which these parasites exploit nutrients available within host cells and alter the biochemical composition of host tissues.

Interactions of Ameson michaelis with its blue crab host contributed to significant alterations in the biochemical constituents of host tissues. Modifications in skeletal muscle protein and carbohydrate metabolism were ultimately reflected in substantial variations in hemolymph composition. Blood osmolality, Cl^- and Na^+ ion levels were decreased with heavy parasitic invasion, while K^+ ion and ninhydrin positive substances (NPS) levels were elevated in both light and heavy infections. Microsporidiosis resulted in a general increase in all hemolymph free amino acids detected except glutamic acid, which declined in concentration in parasitized animals.

Effects of A. michaelis sporogenesis on host tissues were observed by comparing the biochemical composition of thoracic and

cheliped skeletal musculature. Reduced levels of protein and carbohydrate reserves were noted in infected thoracic relative to cheliped skeletal muscle while the opposite trend was observed for tissue free amino acids (NPS). The concentration of nine of the sixteen amino acids detected were not significantly altered with infection. Skeletal muscle glutamate, proline, glycine, alanine and arginine levels declined in parasitized animals while taurine and tyrosine levels were elevated.

Microsporidan infection produced significant lactic acid accumulation in the hemolymph, thoracic muscle and hepatopancreas of parasitized blue crabs. Lactate concentrations reached six to seven times their normal levels in hemolymph and muscle, and four times the control value in the hepatopancreas. Parasitized animals also evidenced reduced blood glucose levels during the terminal stages of microsporidan infection.

Until recently, biochemical investigations of the metabolic processes of the Microsporida have suffered as a result of insufficient numbers of the different parasite stages and inadequate methods for the in vitro cultivation and maintenance of these organisms. Microsporidan infection in the blue crab results in the production of large numbers of parasites which can be easily recovered and induced to hatch. We have investigated the carbohydrate metabolism of pure populations of microsporidan sporoplasms during brief periods of maintenance in extracellular culture media.

Ameson michaelis sporoplasms readily utilized glucose when maintained extracellularly in Medium 199 supplemented with 3 mM ATP. A declined in exogenous glucose was followed by the appearance of substantial quantities of both lactic and, to a lesser extent, pyruvic acids. The production of lactate by isolated microsporidan parasites may represent a significant contribution to the accumulation of this metabolite in the thoracic skeletal muscle of infected animals.

Cells deprived of ATP readily lost their ultrastructural integrity, did not consume exogenous glucose, and produced only reduced quantities of pyruvate and barely detectable levels of lactate. In the presence of 10 mM NaF, a potent glycolytic inhibitor, sporoplasms did not utilize the glucose supplied in the medium nor did they evolve substantial quantities of glycolytic end-products.

The rate of glucose transport into microsporidan cells proceeded more rapidly at low (0.5 mM) rather than high (5.5 mM) substrate concentrations. Glucose uptake by A. michaelis sporoplasms displayed sensitivity toward known inhibitors (i.e., 1 mM ouabain and 0.5 mM amiloride) of sodium transport. The relative importance of Na^+-dependent glucose transport appeared to vary with substrate concentration.

Chapter 1

Overview

Prefatory Comments

Intracellular parasitism represents an extreme of ecological specialization; nothing less than an intact living host cell will suffice to satisfy the environmental requirements and nutritional needs of an intracellular parasite (Trager, 1974). The Microsporida are a large group of highly specialized obligate intracellular protozoan parasites. Although they are best known as parasites of arthropods (Weiser, 1963; 1976) and fish (Sinderman, 1970; Canning, 1976; 1977), microsporidans have been implicated as the etiological agent of various pathologies evidenced in birds (Kemp and Kluge, 1975), amphibians and reptiles (Canning et al., 1964; Schuetz et al., 1978) and a variety of mammals including rodents (Weiser, 1965), carnivores (Vávra et al., 1971), ungulates (Khanna and Iyer, 1971) and primates, including man (Lainson et al., 1964; Viting, 1969; Canning, 1975). Despite their wide distribution in nature and their devastating effect on a variety of animal populations, relatively little is known about the physiological characteristics of these parasites and the infections which they produce.

1. Microsporidan Development

The microsporidan life cycle is comprised of two distinct aspects; (i), the vegetative phase or merogony (commonly designated "schizogony"), during which parasite numbers increase rapidly and the infection is disseminated through the host tissues; and (ii), sporulation, during which supplemental parasite multiplication occurs and mature spores are produced.

Schizogony includes both binary and multiple fission. However, the former is less common because in the microsporidan cell cycle, karyokinesis takes place repeatedly before cytokinesis with the resulting production of plasmodial stages of various shapes and sizes (Vávra, 1976). These plasmodial stages subsequently segregate into individual cells which develop into spores, thus completing the developmental cycle. Stages of the vegetative phase of the life cycle (also termed merogonial stages or trophozoites) include: (i), sporoplasm or sporoplast -- the stage which intially invades host cells; and (ii), schizont (or meront) -- the stage which multiplies rapidly and through which the infection is spread to target host tissues. The life cycle stages of the sporulation phase include: (i), sporont or sporoblast mother cell -- the stage of additional parasite multiplication (sporogony) and of possible involvement in autogamic gene recombination; and (ii), sporoblast -- the stage experiencing marked morphogenesis leading to the production of the mature spore stage (spore morphogenesis = sporogenesis).

The life cycle stages of the vegetative and sporulation phases are significantly different not only in their ultrastructure but also in their relationships to the host cell (Vávra, 1976). The relative multiplicative importance of schizogony and sporogony vary among the different microsporidans. In some species, the schizogony sequence is relatively short and the parasite multiplies extensively during sporogony (e.g., Pleistophora). In other forms, the vegetative phase is the dominant multiplicative period and the sporogonic phase is relatively brief with the number of spores originating from a single sporont being strictly

limited (e.g., Nosema, Encephalitozoon, Thelohania and others) (Vávra, 1976).

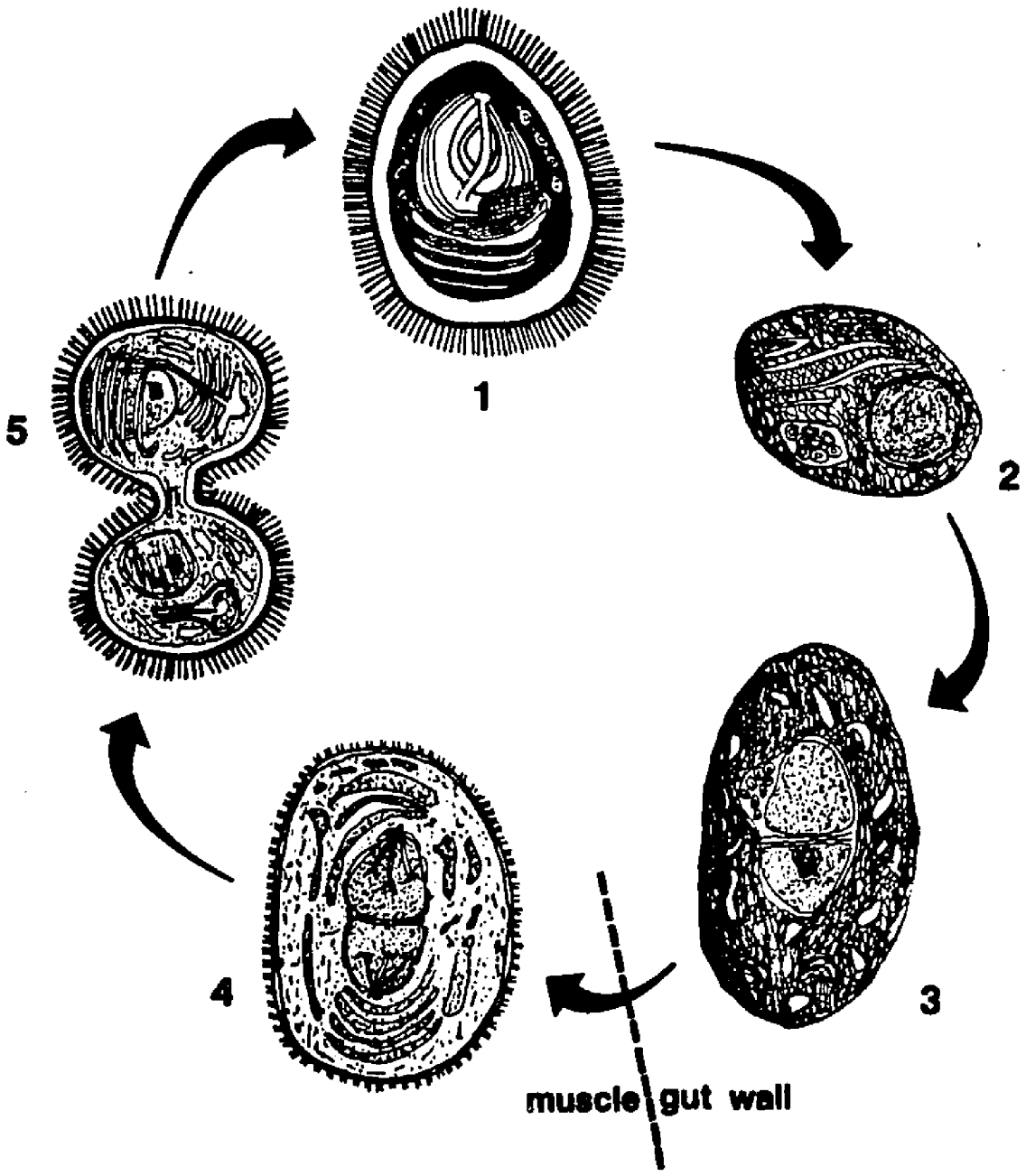
2. Ameson michaelis in Callinectes sapidus

Microporidiosis is responsible for extensive muscle infections in all of the commercially important crustaceans of the Gulf Coast area. Ameson michaelis (=Nosema michaelis) establishes extensive muscle infections in the blue crab, Callinectes sapidus, resulting in death of the host within 20-30 days after initial exposure to infective spore material. Ultrastructural examination of infected muscle tissue indicates the production of large numbers of infective spores (up to 10^9 parasites/gm infected muscle).

When spores are ingested by a suitable host or are subjected to the appropriate in vitro conditions, germination of the spore stage results (see Fig. 1). The spore contains an extensive, coiled invasion tube which injects the infective spore-cell (sporoplasm or sporoplast) into the host gut epithelium (Weidner, 1970; 1972). The injected sporoplasm then develops into a schizont by growth and differentiation of cytoplasmic organelles. Ameson michaelis schizonts develop and multiply particularly within cells of the midgut submucosa layer. Schizont-infected cells lie in close proximity to the hemocoel of the blue crab. It is currently thought that liberated schizonts are carried by the crab's open vascular system to the neighboring skeletal muscle where vegetative invasion and sporogenesis ensues (Weidner, 1970).

Figure 1-1. Life cycle of Ameson michaelis (Microsporida) in the blue crab, Callinectes sapidus. Mature spores (1) are ingested by the host and are primed to hatch as they traverse the alimentary tract. The infective spore-cell or sporoplasm (2) is injected into the gut epithelium. Schizonts or trophozoites (2 & 3) are found within cells of the midgut submucosa layer. Sporogenesis begins with the invasion of thoracic skeletal musculature. The sporont (4) divides into sporoblasts (5) which differentiate into mature spores (1).

Life Cycle of Ameson michaelis



(after Weidner, 1970)

Parasite development within the myofibrils of skeletal muscle proceeds rapidly and is accompanied by the disorder, disassembly and final disappearance of the component myofilaments (Weidner, 1970). Spore development begins when the invading diplokaryotic schizont differentiates into the sporont or sporoblast mother cell. After repeated nuclear divisions, the sporont undergoes delayed cytokinesis and a series of sporoblast progeny develop (Weidner, 1970). Sporoblasts then undergo rapid morphogenesis and differentiation into mature spores. The sporoblasts become encapsulated with a chitinous extramembranous layer (the endospore) which increases in thickness and is delimited by a pellicle coat studded with microtubules (the exospore). Simultaneously, within the developing sporoblast, the cytoplasmic organelles associated with the invasion apparatus of the mature spore (i.e., the polar filament and polaroplast) begin to appear. With the completion of sporoblast differentiation and maturation, the resulting spores are available for entrance into a new host animal.

Ameson michaelis occurs sporadically in the blue crab along the Gulf Coast. Adult crabs are infected in the Lake Pontchartrain/Lake Borgne area of southeastern Louisiana but seldom in large numbers. Professional crab fishermen from this area estimate the level of incidence of noticeably infected crabs to be less than 1% on a year-round basis (Overstreet, 1978). However, in warmer inshore areas, Ameson michaelis infections usually reach a peak of 4-5% incidence in August-September (Julian King, personnal communication).

3. Description of Intent

Previous studies concerning the Microsporida have dealt almost exclusively with descriptions of morphology and life cycles. Relatively little is presently known about the physiological characteristics of these parasites or of their interactions with host cell elements. Until recently, biochemical investigations of the metabolic processes of the Microsporida have suffered as a result of insufficient numbers of the different parasite stages and inadequate methods for the *in vitro* cultivation and maintenance of these organisms. However, microsporidan infection in the blue crab results in the production of large numbers of parasites. Since *Ameson michaelis* spores can be easily recovered and induced to hatch (Weidner, 1972; 1976), the opportunity now exists to investigate pure populations of these intracellular parasites during brief periods of maintenance in extracellular culture media.

The goal of the present research has been to investigate the biochemical and physiological activities of *A. michaelis* during growth within the blue crab, *Callinectes sapidus*. The ultimate objectives have been to identify some of the mechanisms by which these intracellular parasites exploit nutrients available within host cells and alter the biochemical composition of host tissues. Additionally, this work represents the first study concerning the physiological activities of pure populations of isolated, extracellular microsporidan parasites.

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Chapter 2

Microsporidan-Induced Alterations in the Biochemical Composition of Blue Crab Host Tissue

INTRODUCTION

Parasitism frequently results in the significant alteration of host cell metabolism. Disturbances in the biochemical composition of host tissues accompanying protozoan infection are well documented (see von Brand, 1973). The concentration of various inorganic constituents in the blood plasma have been shown to vary in several malaria (Zwemer et al., 1940; Overman et al., 1949; Sadun et al., 1965; Sherman and Tanigoshi, 1971) and in the later stages of trypanosomiasis (Zwemer and Culbertson, 1939; Ikejiani, 1946 a,b). Parasite-induced perturbations in host carbohydrate metabolism may result in abnormalities in blood sugar levels and tissue polysaccharide reserves (Mercado, 1952; Mercado and von Brand, 1954; von Brand and Mercado, 1956; Manwell and Stone, 1968; Moon et al., 1968; Sanchez and Dusanic, 1968; and others). Similarly, changes in the protein and free amino acid levels of host tissues frequently occur during protozoan infection (Sherman and Mudd, 1966; Ormerod, 1967; Siddiqui and Trager, 1967; Wang and Moeller, 1970).

Among parasitic protozoans, the microsporidan Ameson michaelis is of general interest because it accomplishes massive invasion of host cells, proceeds rapidly through sporogenesis in host muscle and inflicts widespread tissue damage. A. michaelis sporogenesis takes place in the blue crab sarcoplasm. After entry into myofibrils, schizonts differentiate into sporonts with subsequent multiplication into sporoblasts (Weidner, 1970). The colonies of sporoblasts in the sarcoplasm provide

an apparent molecular chaos which results in the disorientation and eventual loss of the highly organized myofibrillar arrangement of the host muscle (Weidner, 1970). As the infection proceeds to its terminal stages, the host skeletal musculature is largely replaced by parasite spores. In the present study, we have characterized the physiological consequences of microsporidan infection in the blue crab as a function of the altered biochemical composition of host skeletal muscle, hemolymph and hepatopancreas tissues.

MATERIALS AND METHODS

Adult intermolt blue crabs, Callinectes sapidus, infected with Ameson michaelis were collected from Lake Pontchartrain in the vicinity of Irish Bayou, Louisiana. Additionally, non-infected blue crabs were simultaneously collected from the same waters for comparison with infected individuals. The animals were transported to the laboratory over ice and sampled immediately. Small sections of thoracic skeletal muscle tissue were removed from infected blue crabs and pressed between two glass slides. This preparation was examined microscopically to determine the extent of parasite development in individual animals -- blue crabs exhibiting $\leq 10^3$ parasites/gm tissue were considered to be lightly infected; $>10^3$ parasites/gm tissue constituted a heavy infection.

Hemolymph Analyses

Osmolality and Ion Analyses

The membrane at the base of the pereiopod (swimming leg) was blotted dry and a 300-400 µl sample of hemolymph withdrawn into snap-cap microcentrifuge tubes using unheparinized capillary tubes. Blood samples were centrifuged immediately at 10,000 g for 4 min. to remove any particulate material. The osmolality of hemolymph and seawater samples was determined with a WESCOR Vapor Pressure Osmometer. Hemolymph samples were diluted with deionized water for ion analyses. Chloride ion concentrations were determined with an Aminco Chloridometer. Sodium and potassium levels were determined with a Coleman Flame Photometer.

Ninhydrin Positive Substances

Additional aliquots of hemolymph were collected for ninhydrin positive substances (NPS) determinations. Protein was precipitated from the hemolymph by diluting each sample with 5, sulfosalicylic acid to a final concentration of 10%. Following centrifugation (10,000 g; 4 min), the NPS level of the supernatant was determined colorimetrically by the method of Rosen (1957). Leucine was used as the standard. Furthermore, an amino acid profile of the protein-free supernatant was determined with a Beckman Automatic Amino Acid Analyzer.

Glucose Analyses

Hemolymph samples (0.2 ml HL in 2.0 ml distilled water) were deproteinized by the addition of 1.0 ml 0.3 N BaOH and 1.0 ml 5% $ZnSO_4$.7 H_2O. Following centrifugation (10,000 g; 4 min), the glucose concentration of the protein-free supernatant was quantified colorimetrically by the glucose oxidase-peroxidase method (Raabo and Terkildson, 1960 as modified by Sigma Chemical Co., Kit No. 510).

Lactate Analyses

Following collection, 0.2 ml hemolymph samples were immediately transferred into microcentrifuge tubes containing 0.4 ml cold 8% perchloric acid. The tubes were agitated and returned to an ice bath for 5 min to assure complete precipitation of blood proteins. The mixture was subsequently centrifuged (10,000 g;15 min) and the lactate concentration of the protein-free supernatant was quantified enzymatically by the Sigma method for the ultraviolet determination of lactic

acid at 340 nm (Sigma Chemical Co., Kit No. 826-UV).

Skeletal Muscle and Hepatopancreas Analyses

Protein, Ninhydrin Positive Substances and Carbohydrate Concentrations

Skeletal muscle tissue was removed from the thoracic region and the pereiopods (including the chela, walking legs and paddles). Dissected tissue was flash frozen overy dry ice, lyophilized to dryness and subsequently ground to a uniform particle size in a Thomas-Wiley tissue grinder. Ground tissue was stored frozen (-20°C) in one dram glass vials with cork stoppers and when needed was removed from the freezer and warmed to room temperature in a dessicator to prevent any possible water adsorption. Protein was extracted by refluxing 10 mg of the ground tissue in 1 N NaOH for 30 min at 100°C. The protein content of the tissue extracts was determined by the method of Lowry et al. (1951). Bovine serum albumen was used as the reference standard. Additional aliquots of the NaOH extracts were analyzed for carbohydrate concentration (Montgomery, 1957). Glycogen was used as the standard. Hepatopancreas samples were prepared and analyzed for their protein and carbohydrate levels in a similar manner.

For the determination of tissue ninhydrin positive substances (NPS), 10 mg of ground skeletal muscle tissue was incubated in 10% 5, sulfosalicylic acid (48 hr; 22°C) to precipitate all proteins and allow for the complete leaching of free amino acids from the tissue. Samples were subsequently centrifuged (10,000 g; 15 min) and supernatant NPS was determined (Rosen, 1957). Leucine was used as the standard. An amino

acid profile of additional quantities of the NPS digest was determined with a Beckman Automatic Amino Acid Analyzer.

Lactate Concentrations

Thoracic skeletal muscle and hepatopancreas tissues were removed from animals, flash frozen over dry ice and weighed. Individual samples were homogenized in ten times their weight of cold 8% perchloric acid. Homogenates were transferred to microcentrifuge tubes and returned to an ice bath for 5 min to allow complete precipitation of tissue proteins. Samples were centrifuged (10,000 g; 5 min) and the lactate concentration of the protein-free supernatant was quantified enzymatically by the Sigma method for the ultraviolet determination of lactic acid at 340 nm (Sigma Chemical Co., Kit No. 826-UV).

RESULTS

Hemolymph Profile

Callinectes sapidus infected with the microsporidan parasite *Ameson michaelis* were collected from Lake Pontchartrain, Louisiana during August and September, 1978 and 1979. Salinity at the time of collection was 2-6‰S, water temperature varied from 29-34°C and the dissolved oxygen content ranged between 5-7 mg/l. Water under these temperature and salinity conditions was 70-95% saturated with dissolved oxygen.

Parasite infection produced marked alterations in blue crab hemolymph composition. Blood osmolality, Cl^- and Na^+ ion levels were decreased with infection while K^+ ion and ninhydrin positive substances (NPS) levels were elevated (Table 2-1 and 2-2). Identical trends were exhibited by animals collected during the 1978 and 1979 seasons. Light infection generally produced insignificant changes in hemolymph osmolality, Na^+ and Cl^- ion concentrations ($P>0.05$) while K^+ ion and NPS levels were significantly altered ($P<0.05$). Blood K^+ values were 25% ($\sim$1.5-2.0 mM/l) higher than control concentrations. NPS levels were nearly twice (or approximately 3-4 mM/l higher than) those of normal animals.

Heavy parasite invasion was accompanied by significant alterations in all blood constituents assayed ($P<0.01$). Hemolymph osmolality decreased by 10% ($\sim$60-70 mOsm/kg water) and Cl^- and Na^+ ion values each declined by 15% (approximatley 50 mM/l) of their control levels. K^+ ion concentrations rose 60% or nearly 5 mM/l

Table 2-1. Effects of microsporidan infection on the hemolymph composition of blue crabs. Values presented are for blue crabs collected from Lake Pontchartrain, Louisiana during the summer of 1978 (top line of each entry) and 1979 (bottom line of each entry). Salinity at the time of collection was 2-4‰S (1978) and 2-6‰S (1979). All hemolymph values are given as the mean ± 95% confidence interval; sample size is indicated in parentheses.

	Normal	Infected	
		Light	Heavy
Osmolality	663.3 ± 17.5(10)	641.3 ± 6.7(10)	594.2 ± 10.4(10)
mOsm/kg H_2O)	671.6 ± 21.5(5)	639.5 ±10.1(5)	594.3 ± 9.9(5)
Cl^-	327.7 ± 8.1(10)	314.5 ±13.6(10)	278.5 ± 8.6(10)
mM/l	328.0 ± 1.7(5)	317.0 ± 5.4(5)	275.0 ± 10.5(5)
Na^+	304.7 ± 8.6(10)	290.5 ± 5.9(10)	259.0 ± 13.0(10)
mM/l	304.3 ± 6.8(5)	294.7 ± 6.8(5)	261.0 ± 12.5(5)
K^+	7.6 ± 0.8(10)	9.7 ± 0.4(10)	12.2 ± 1.5(10)
mM/l	8.2 ± 0.5(5)	9.9 ± 0.5(5)	13.3 ± 1.1(5)
NPS	4.1 ± 0.8(10)	8.3 ± 0.6(10)	12.4 ± 1.9(10)
mM/l	5.4 ± 0.6(5)	8.3 ± 1.1(5)	12.6 ± 2.4(5)

Table 2-2. Comparison of hemolymph profile from normal and infected blue crabs. Significance refers to t-test comparisons between values. NS means not significant at P<0.05 level. The first line of each comparison is for 1978 data; the second entry is for 1979 values.

	mOsm	Cl^-	Na^+	K^+	TNPS
Normal vs. Light	NS	NS	NS	P<0.05	P<0.01
Infection	NS	P<0.05	NS	P<0.05	P<0.05
Normal vs. Heavy	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
Infection	P<0.01	P<0.01	P<0.01	P<0.01	P<0.01
Light vs. Heavy	P<0.01	P<0.05	P<0.05	NS	P<0.05
Infection	P<0.01	P<0.01	P<0.05	P<0.05	NS

above normal while NPS values were 2.5-3 times or approximatley 8 mM/l higher than those of control crabs. Additionally, heavy infection resulted in significantly greater disturbances in all hemolymph constituents than more light parasite invasions.

In comparison to normal animals, the amino acid profile of hemolymph collected from infected blue crabs was substantially altered (Table 2-3). Nine amino acids and ammonia were detected in appreciable quantities. Parasitic infection resulted in general elevation of all free amino acids except glutamic acid. Infected animals exhibited a two- or three-fold increase in hemolymph aspartic acid and threonine levels ($P<0.05$) as well as arginine, taurine and NH_3 concentrations ($P<0.01$). More dramatic increases were seen in blood alanine and proline levels. Alanine exhibited a 7-fold increase with infection, a rise of $\sim$0.400 µmoles/ml; proline showed an 8-fold increase, a rise of $\sim$0.470 µmoles/ml. Conversely, only a moderate rise of about 50% ($\sim$0.400 µmoles/ml) was observed in the glycine concentration. Glutamic acid decreased 60% ($\sim$0.080 µmoles/ml) and was the only amino acid to decline in concentration in infected animals.

Skeletal Muscle Profile

Microsporidan infection generated significant modifications in host muscle protein, free amino acid (NPS) and carbohydrate concentrations. Due to the wide variation in the level of these biochemical constituents among different blue crabs, data were analyzed on a single animal basis as the % difference between the thoracic and

Table 2-3. Amino acid profile of normal and infected blue crab hemolymph. All amino acid values are given as the mean ± 95% confidence interval; sample size is indicated in parentheses. Significance refers to t-test comparisons between values. NS means not significant at $P<0.05$ level.

	Normal	Infected	Significance
µmoles/ml	(n=4)	(n=6)	
tau	0.380 ± 0.160	1.060 ± 0.264	P<0.01
asp	0.156 ± 0.005	0.357 ± 0.079	P<0.05
thr	0.027 ± 0.011	0.087 ± 0.021	P<0.05
ser	0.084 ± 0.014	0.118 ± 0.041	NS
glu	0.200 ± 0.013	0.117 ± 0.022	P<0.01
pro	0.071 ± 0.010	0.544 ± 0.129	P<0.01
gly	0.767 ± 0.108	1.176 ± 0.293	P<0.05
ala	0.074 ± 0.008	0.475 ± 0.141	P<0.01
arg	0.162 ± 0.018	0.518 ± 0.049	P<0.01
NH_3	3.235 ± 0.681	5.947 ± 0.566	P<0.01

cheliped muscle value for each constituent. The rationale for this presentation was dictated by our knowledge that the thoracic region is the first and most heavily infected of the crab skeletal musculature. Conversely, the cheliped muscle appears secondarily infected and the number of parasites present in the claw tissue is often minimal until the most terminal stages of the infection. Consequently, a comparison of thoracic and cheliped muscle would maximize the detection of infection-related perturbations in biochemical composition.

Skeletal muscle protein varied between 600-750 μg/mg dry tissue. Both light and heavy infection produced marked and significant decreases in thoracic muscle protein (Table 2-4 and 2-5). Light parasite invasion resulted in a decline of 6.0 $\pm$ 1.2% in thoracic relative to cheliped muscle protein. Heavy infection produced a more dramatic decrease (12.5 $\pm$ 0.7%) in thoracic muscle protein. Changes incurred during light and heavy infections were significantly different from the normal condition ($P<0.01$). Additionally, light and heavy infection also differed statistically ($P<0.01$) in their effect on host muscle protein levels.

Skeletal muscle free amino acid levels ranged from 0.8-1.3 μmoles/mg dry tissue. In infected animals, thoracic muscle NPS concentrations were significantly elevated with respect to cheliped levels (Table 2-4 and 2-5). Light infection resulted in a 11.5 $\pm$ 3.5% increase in thoracic versus cheliped muscle NPS; heavy infection produced a more dramatic rise in thoracic muscle free amino acids (27.0 $\pm$ 5.5%). In comparison with normal blue crabs, parasite invasion provided significantly greater alterations in the NPS ratio

Table 2-4. Effect of microsporidan infection on the skeletal muscle composition of blue crabs. Data are presented as the % difference in protein, free amino acid (NPS) and carbohydrate levels of the thoracic vs. chelaped skeletal muscle. Arrows indicate whether values for thoracic muscle were higher (↑) or lower (↓) than those of chelaped muscle. All values are given as the mean ± the 95% confidence interval; sample size is indicated in parentheses.

	Normal	Infected	
		Light	Heavy
	(n=6)	(n=10)	(n=6)
	% Difference Thoracic vs. Chelaped Muscle		
Protein	↑0.97 ± 0.24	↓5.98 ± 1.18	↓12.45 ± 0.65
NPS	↑ 2.60 ± 0.93	↑11.49 ± 3.46	↑27.03 ± 5.45
Carbohydrate	↑ 6.00 ± 0.71	↓ 4.65 ± 1.21	↓18.07 ± 3.04

Table 2-5. Comparison of the biochemical composition of skeletal muscle tissue in normal and infected blue crabs. Significance refers to t-test comparisons between values.

	Protein	NPS	Carbohydrate
Normal vs. Light Infection	P<0.01	P<0.05	P<0.01
Normal vs. Heavy Infection	P<0.01	P<0.01	P<0.01
Light vs. Heavy Infection	P<0.01	P<0.05	P<0.01

of thoracic and cheliped muscle.

An amino acid profile of the NPS digest revealed considerable changes in several of the sixteen amino acids detected (Table 2-6). A decrease in infected thoracic muscle alanine, arginine, glutamic acid, glycine and proline concentrations was observed. Arginine levels dropped more than 65% or ∿0.220 μmoles/mg dry tissue. This represented the most dramatic decrease of all the amino acids surveyed. Alanine and proline values exhibited more moderate declines of nearly 50% or ∿0.090-0.100 μmoles/mg dry tissue. Glutamic acid and glycine levels displayed smaller decreases of approximately 30% (or ∿0.030 and 0.150 μmoles/mg dry tissue, respectively) in concentration. Conversely, infected muscle taurine and tyrosine values were twice those of normal animals. With infection, taurine increased from approximatley 0.100 to 0.200 μmoles/mg dry tissue while tyrosine levels went from ∿0.011 to .022 μmoles/mg dry tissue. Muscle aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine and valine concentrations remained virtually unchanged from their control values ($P>0.05$). Tissue NH_3 levels rose insignificantly with infection ($P>0.05$).

Skeletal muscle carbohydrate concentrations varied from 20-35 μg/mg dry tissue. Both light and heavy infections produced significant reductions in thoracic relative to cheliped muscle carbohydrate level (Table 2-4 and 2-5). Light infection resulted in a $4.7 \pm 1.2\%$ decline in thoracic versus cheliped muscle carbohydrate; heavy infection was associated with additional decreases in thoracic muscle carbohydrate ($18.1 \pm 3.0\%$). Alterations in muscle carbohydrate

Table 2-6. Amino acid profile of thoracic skeletal muscle from normal and infected blue crabs. All amino acid values are given as the mean ± 95% confidence interval; sample size is indicated in parentheses. Significance refers to t-test comparisons between values. NS means not significant at P<0.05 level.

	Normal	Infected	Significance
μmoles/mg	(n=4)	(n=6)	
tau	0.096 ± 0.019	0.193 ± 0.038	P<0.05
asp	0.029 ± 0.003	0.029 ± 0.003	NS
ser	0.043 ± 0.008	0.044 ± 0.015	NS
glu	0.095 ± 0.011	0.061 ± 0.012	P<0.05
pro	0.189 ± 0.027	0.095 ± 0.034	P<0.05
gly	0.508 ± 0.049	0.352 ± 0.053	P<0.05
ala	0.192 ± 0.032	0.106 ± 0.027	P<0.05
val	0.015 ± 0.001	0.014 ± 0.002	NS
met	0.011 ± 0.004	0.010 ± 0.004	NS
ile	0.009 ± 0.004	0.010 ± 0.004	NS
leu	0.030 ± 0.023	0.016 ± 0.009	NS
tyr	0.011 ± 0.001	0.022 ± 0.003	P<0.05
phe	0.008 ± 0.001	0.009 ± 0.004	NS
his	0.017 ± 0.004	0.015 ± 0.005	NS
lys	0.021 ± 0.003	0.021 ± 0.007	NS
arg	0.319 ± 0.037	0.099 ± 0.030	P<0.01
NH_3	0.116 ± 0.022	0.130 ± 0.028	NS

concentrations observed during parasitic infection were statistically different from the control condition ($P<0.01$).

Additionally, carbohydrate and protein concentrations were observed for hepatopancreas tissue isolated from infected blue crabs. Parasitic infection produced no significant changes in hepatopancreas carbohydrate (from 15.9 $\pm$ 0.5 to 15.2 $\pm$ 0.9 µg/mg dry tissue, $P>0.05$) or protein (from 250.5 $\pm$ 11.7 to 223.0 $\pm$ 10.4 µg/mg dry tissue, $P>0.05$) levels.

Lactate Levels

Microsporidan infection caused a marked increase in lactic acid levels of hemolymph, skeletal muscle and hepatopancreas host tissues (Table 2-7 and 2-8). Hemolymph samples exhibited the lowest control lactic acid levels. Light infection affected a four-fold increase (approximatley 0.300 mg/ml) in crab hemolymph lactate concentration. Heavy infection produced additional lactate accumulation to 0.722 $\pm$ 0.032 mg/ml. Light parasite invasion resulted in a two-fold ($\sim$0.250 mg/g) rise in thoracic muscle lactate levels whereas, heavy infections yielded a six-fold increase in muscle lactate to 1.237 $\pm$ 0.276 mg/g. Control lactate values were higher for the hepatopancreas than for either of the other normal tissues. A significant 65% ($\sim$ 0.200 mg/g) elevation of hepatopancreas lactate concentrations accompanied light infection while heavy infection produced a 4.5-fold increase in normal lactate levels to 1.465 $\pm$ 0.322 mg/g.

Additionally, blue crabs subject to microsporidan invasion

Table 2-7. Lactate concentration in the hemolymph, thoracic skeletal muscle and hepatopancreas of blue crabs. All values are given as the mean ± the 95% confidence interval; sample size is indicated in parentheses.

	Normal	Infected	
		Light	Heavy
	(n=6)	(n=6)	(n=6)
Hemolymph mg/ml	0.110 ± 0.050	0.441 ± 0.036	0.722 ± 0.032
Thoracic Muscle mg/g	0.228 ± 0.051	0.548 ± 0.022	1.237 ± 0.276
Hepatopancreas mg/g	0.314 ± 0.069	0.512 ± 0.062	1.465 ± 0.322

Table 2-8. Comparison of lactate concentrations in normal and infected blue crabs. Significance refers to t-test comparisons between values.

	Hemolymph	Thoracic Muscle	Hepatopancreas
Normal vs. Light Infection	P<0.01	P<0.01	P<0.05
Normal vs. Heavy Infection	P<0.01	P<0.01	P<0.01
Light vs. Heavy Infection	P<0.01	P<0.05	P<0.01

evidenced hypoglycemia in the terminal stages of the infection. Hemolymph glucose levels declined from a control value of 66.3 $\pm$ 2.8 mg/100 ml to 51.1 $\pm$ 3.5 mg/100 ml. The data represent a significant difference ($P<0.01$) between normal and infected animals.

DISCUSSION

Despite the considerable amount of literature which exists pertaining to the Microsporida, limited information is available concerning the physiological changes incurred by host animals as a result of microsporidan infection. It is reasonable to assume that during infection, the host cell environment influences the metabolic activities of an intracellular parasite. In the present study, we have shown that the interactions of the microsporidan parasite Ameson michaelis with its blue crab host, Callinectes sapidus, have their counterpart in somatic (largely thoracic skeletal muscle tissue) alterations which are ultimately reflected in biochemical changes in the host blood.

The estuarine environment of the blue crab is subject to rapid fluctuations in osmotic and ionic composition (see Stickle and Howey, 1975). Callinectes sapidus has been shown to exhibit considerable capacity for osmotic and ionic regulation over widely fluctuating salinity regimes (Findley and Stickle, 1978). The capacity for water and salt regulation varies with a number of exogenous and endogenous factors and among the latter we may include parasitism. We have observed that low salinity, high temperature and reduced oxygen content appear to be an optimal combination for the establishment of Ameson michaelis in the blue crab. The same combination of factors also seems to be particularly stressful for the regulatory abilities of crabs (see Kinne, 1971).

The hemolymph composition of control blue crabs used in this

study is consistent with that previously described for crabs maintained at extremely low salinities (Mangum and Amende, 1972; Cameron, 1978). As a consequence of parasitic infection, significant alterations are observed in hemolymph osmotic and ionic composition. Blood osmolality, Cl^- and Na^+ ion concentrations decrease with infection, while K^+ ion and free amino acid (NPS) levels are elevated. The observed changes may be partially attributed to the release of ions and free amino acids as a result of host cell lysis. However, the drop in blood osmolality during heavy infection cannot be accounted for by cell lysis because the extracellular and intracellular fluid compartments are isosmotic. It is significant to note, however, that statistical alterations in all of the blood constituents surveyed are seen only during heavy microsporidan infection. Severe parasite invasion results in the production of large numbers of mature spores and the concomitant disruption of host skeletal muscle tissue (Weidner, 1970). Animals supporting more moderate muscle infections (proportionally larger number of young sporoblast stages present) do not evidence altered hemolymph profiles. Presumably during light infection, blue crabs are sufficiently competent to meet the challenge of initial cell lysis. However, their regulatory abilities may become inadequate to accomodate the massive quantities of host cell damage accompanying the terminal infection.

Significant alterations in host blood composition have been observed in several protozoan infections. Blood K^+ concentrations are elevated in many malaria (Zwemer et al., 1940; Velick and Scudder, 1940; McKee et al., 1946; Overman et al., 1950; Sherman and Tanigoshi, 1971)

and in the later stages of trypanosomiasis (Zwemer and Culbertson, 1939; Ikeijiani, 1946a,b). There are likely a number of factors contributing to the increased potassium levels (von Brand, 1973). While a large fraction is undoubtedly derived from host cell lysis, it seems that in malaria at least, changes in membrane permeability of fragile infected erythrocytes as well as nonparasitized host blood cells promote potassium leakage into the plasma (Overman et al., 1949). In the past, it had been postulated that blood potassium accumulation, in addition to other ion imbalances, might lead to fatal metabolic disturbances in malaria (Overman et al., 1949) and trypanosomiasis (Zwemer and Culbertson, 1939). However, this is presently thought not to be the case since K^+ ion accumulation is well below the toxic level (see Maegraith, 1949). Additionally, this also appears to be true for blue crabs subject to severe microsporidan infection since elevated K^+ ion concentrations remain within the range of values typically detected in these animals at higher ($\sim$30 ‰S) environmental salinities (Findley and Stickle, 1978).

The sodium level of blood plasma is sometimes, but not always, lowered in protozona infection (Overman et al., 1949; Sadun et al., 1965; Sherman and Tanigoshi, 1971). On the other hand, plasma chloride levels typically remain unchanged in malaria (Sadun et al., 1965) and trypanosomiasis (Moon et al., 1968).

Microsporidan infection in the blue crab results in a substantially altered hemolymph free amino acid profile. In crustaceans, glycine, alanine, glutamic acid, proline, serine, ornithine and taurine typically

comprise 70-90% of the blood free amino acid pool, with the relative concentrations of the different amino acids varying characteristically among species (Camien et al., 1951; Stevens et al., 1961; Vincent-Marique and Gilles, 1970; Lynch and Webb, 1973a). In salinities of 16.5-21.0 ‰S, glycine, taurine, alanine, proline and arginine constitute 72-90% of the total free amino acids in the serum of mature Callinectes sapidus (Lynch and Webb, 1973a). At extremely low salinities (2-4 ‰S), only nine amino acids and ammonia are detected in appreciable quantities and the relative concentrations of proline and alanine appear to be reduced while the levels of glutamic and aspartic acids are proportionally increased.

In the blue crab, microsporidan infection contributes to a general elevation of all blood free amino acids except glutamic acid. It seems reasonable to assume that the observed increases in amino acid levels are principally derived from host muscle proteolysis. However, parasite invasion may promote modifications in the permeability of muscle cell membranes. As a result, intracellular amino acids might leak to the extracellular spaces. Such a mechanism is know to be operative in osmotic and volume regulation in euryhaline animals (see Fugelli, 1967; Gilles and Schoffeniels, 1969; Vincent-Marique and Gilles, 1970).

The accumulation of amino acids in the body fluids of infected animals may indicate that the capacity for hydrolysis of host cell protein is greater than that for incorporation of the resultant amino acids into parasite protein (see Moulder, 1962). Therefore, the

observed decline in glutamic acid from the hemolymph of infected blue crabs may indicate its significant uptake by developing parasites. Similarly, those amino acids which display only moderate increases in concentration is parasitized animals (i.e., glycine, aspartic acid and serine) may also be heavily utilized. In fact, an acid hydrolysate of Ameson michaelis spores indicates the presence of large quantities of glutamic acid, glycine, aspartic acid and serine, in addition to significant amounts of tyrosine (Weidner, unpublished data).

Comparative data on parasite-induced alterations in the plasma amino acid levels of host animals are limited. The hemolymph amino acid profile of Trypanosoma rangeli-infected reduviid bugs displays a general rise in amino acid concentrations; however, certain amino acids decline in concentration (Ormerod, 1967). Sherman and Mudd (1966) found no distinct changes in the plasma free amino acids of ducks parasitized by Plasmodium lophurae, whereas Siddiqui and Trager (1967) observed rather pronounced increases in several amino acids. Information concerning host blood amino acids during microsporidiosis is limited to a single study of Nosema apis-infected honey bees (Wang and Moeller, 1970). Parasitized bees display smaller amounts of hemolymph amino acids than their non-infected counterparts. Wang and Moeller (1970) suggested that the decrease in plasma amino acids reflects the parasite's utilization of the host blood free amino acid pool. At first glance, our data, describing increased levels of plasma free amino acids, appear to be inconsistent with those of Wang and Moeller which detail the opposite trend. However, this

discrepancy is likely attributable to the use of animals supporting different stages of microsporidan infection. Five to ten day old infected honey bees contain proportionally larger numbers of younger, more metabolically active vegetative and early sporogenic stages than do blue crabs sustaining terminal infections, represented by the abundance of mature spores. Additionally, hones bees, as insects, possess a significantly larger natureally occurring hemolymph free amino acid pool (∿28-65 mM/l), and consequently a more substantial amino acid source for developing parasites, than do blue crabs which are characterized by low hemolymph free amino acid levels (∿4-5 mM/l at 2-4 ‰S).

Microsporidan development within the myofibrils of blue crab skeletal muscle progresses rapidly and is accompanied by the general disorder, disassembly and final disappearance of the component myofilaments (Weidner, 1970). During the terminal stages of the infection, host muscle tissue is largely replaced by mature spores. The initial site of parasite invasion into skeletal muscle is in the thoracic region, in close proximity to the crab midgut which supports early vegetative development. As sporogenesis proceeds, other skeletal muscle masses (i.e., those of the cheliped and other pereiopods) are subsequently infected. The effects of Ameson michaelis sporogenesis on host tissue is observed by comparing the biochemical composition of thoracic and cheliped skeletal musculature. Reduced levels of protein and carbohydrate reserves are detected in thoracic relative to cheliped skeletal muscle while the opposite trend is observed for tissue free amino acid (NPS) concentrations.

Significant decreases in tissue protein and the concomitant increases in free amino acid levels may be attributed to the substantial muscle proteolysis accompanying terminal parasite development. Additionally, the modification of muscle membrane permeability may contribute to changes in the relative abundance of muscle, and consequently hemolymph, free amino acids (see Schoffeniels, 1976). Reductions in muscle carbohydrate reserves (i.e., glycogen) may be ascribed to either the increased metabolic demands of the host muscle and/or the developing parasites.

The free amino acid pool of muscle tissue from Callinectes sapidus maintained in low salinity water (2-4 ‰S) is extremely small relative to that observed at higher salinities (i.e., ∿0.8-1.3 μmoles/mg dry tissue at 2-4 ‰S versus ∿28 μmoles/100 mg wet weight at 50% SW versus ∿42 μmoles/100 mg wet weight at 100% SW, Schoffeniels, 1976). Blue crabs transferred from full-strength to 50% sea water display significnat decreases in virtually all muscle free amino acids (Schoffeniels, 1976). At even lower salinities, further reductions are seen in the free amino acid pool and in particular, in alanine, glycine, proline and taurine concentrations. Comparison of free amino acid profiles of control and parasitized thoracic skeletal muscle indicates that the concentrations of nine of the sixteen amino acids detected are not significantly altered with infection. Glutamic acid, proline, glycine, alanine and arginine levels decline with microsporidan infection, while taurine and tyrosine values are elevated. Modifications in infected muscle

free amino acid levels are, with the exception of tyrosine, reflected in significant changes in their plasma concentration. Tyrosine presents an interesting situation since it is virtually absent from the hemolymph of low salinity animals and is found in substantial amounts in the acid hydrolysate of A. michaelis spores (Weidner, unpublished data). The possibility exists that the uptake of tyrosine by developing parasites from the muscle free amino acid pool is so efficient that this amino acid never accumulates in measureable quantities in the crab blood.

Parasitic infection may result in the deprivation of oxygen to host tissues (see von Brand, 1973). Oxygen insufficiency may result from the increased metabolic demands of host tissues and/or developing parasites or an impaired oxygen delivery system. Under these conditions, anaerobic metabolism becomes increasingly important as a means of energy production (Burke, 1979). In crustaceans, glycolysis appears to be the major functional anaerobic pathway. During hypoxia, significant quantities of pyruvate are converted to lactic acid in crustacean skeletal muscle (Teal and Carey, 1967; Dendinger and Schatzlein, 1973; Phillips et al., 1977; Burke, 1979). Non-parasitized Callinectes sapidus exhibit control levels of hemolymph and thoracic muscle lactic acid concentration which are quite similar to those reported for other crustacean species (Dendinger and Schatzlein, 1973; Burke, 1979). However, blue crab hepatopancreas values are significantly higher than (approximately ten times) those observed for other crabs (Dendinger and Schatzlein,

1973). Elevated lactic acid concentrations may be a reflection of animals collected from a stressed environment (water of low salinity, high temperature and poor oxygen content) or may be an artifact of handling (Dendinger and Schatzlein, 1973).

Microsporidan infection produces significant lactic acid accumulation in the hemolymph, thoracic muscle and hepatopancreas of parasitized blue crabs. Lactate concentrations reach six-seven times their normal levels in hemolymph and muscle, and four times the control value in the hepatopancreas. Since the amount of lactate is always higher in the thoracic muscle than in the hemolymph, it is reasonable to assume that the muscle is the ultimate source of lactate production (Phillips et al., 1977). The hepatopancreas has been reported to possess both reduced activity of glycolytic enzymes and an insignificant level of in vitro lactate production (Dendinger and Schatzlein, 1973; Schatzlein et al., 1973). Therefore, it is likely that increases in hepatopancreas lactate levels represent the clearance of this muscle metabolite from the hemolymph. The role of the hepatopancreas in the metabolism of lactic acid remains unclear. Lactic acid dehydrogenase (LDH) and other enzymes associated with gluconeogenesis from lactate have been reported either to be present in varying levels of activity or to be virtually absent from the hepatopancreas (see Schatzlein et al., 1973; Phillips et al., 1977). However, the absence of enzymatic activity from hepatopancreas homogenates may be the result of its inactivation by proteolytic enzymes released during sample preparation (van Weel, 1970; Schatzlein, et al., 1973).

Plasma glucose concentrations are extremely variable in freshly collected Callinectes sapidus. Control animal blood glucose values were at the high end of the normal range reported for blue crabs taken directly from the natural environment (Dean and Vernberg, 1965a; Lynch and Webb, 1973b). Elevated glucose concentrations may be attributed to handling stress (Telford, 1968a,b), asphyxia (resulting from holding animals out of water) (Kleinholz et al., 1950; Lynch and Webb, 1973b) and high temperatures (Dean and Vernberg, 1965b). Parasitized blue crabs evidence reduced blood glucose levels during the terminal stages of microsporidan infection (a terminal hypoglycemia). Reduced hemolymph glucose may be ascribed to the increased metabolic demands of host cells and/or the developing parasites.

Parasitic protozoans are known to produce significant disturbances in host cell carbohydrate metabolism. Both blood sugar level and polysaccharide reserves may be affected. Terminal hypoglycemia accompanies several malaria infections (Fulton, 1939; Marvin and Rigdon, 1945; Mercado, 1952; Sadun et al., 1965) and trypanosomiasis (Moon et al., 1968; Sanchez and Dusanic, 1968). Liver glycogen content, and to a lesser extent protein level, are lowered in protozoan infection (Mercado and von Brand, 1954; Marciacq and Seed, 1970; von Brand, 1973). Altered liver metabolism is often accompanied by reductions in muscle glycogen (von Brand, 1973). However, Plasmodium and some Trypanosoma infections involve direct parasite invasion of host liver tissue whereas the hepatopancreas of blue crabs is not

subject to infection. Therefore, any disturbances in hepatopancreas carbohydrate metabolism during microsporidan infection are merely a reflection of muscle dysfunction.

Lactic acid is known to accumulate in the blood of protozoan-infected animals (von Brand, 1973). However, in dealing with whole animals studies, it is difficult to ascertain whether elevated lactate concentrations are the result of parasite production or host cell metabolism. In isolated bloodstream forms of pathogenic trypanosomes, aerobic and anaerobic glucose fermentation result in the production of significant quantities of pyruvate and only trace amounts of lactate (Grant and Fulton, 1957; Bowman et al., 1970). Therefore, it seems likely that reported increases in blood lactate are the result of vertebrate host cell metabolism. In malaria, the situation appears more complicated since lactate may be a principle end-product of both parasite and vertebrate host cell glycolysis (Wendel, 1943; Silverman et al., 1944; Speck et al., 1946; Fulton and Spooner, 1956; Bowman et al., 1960; Sherman et al., 1969). In addition to vertebrates, crustaceans have also been shown to produce significant quantities of lactic acid under a variety of stress conditions (i.e., hypoxia and exercise) (Teal and Carey, 1967; Dendinger and Schatzlein, 1973; Phillips et al., 1977; Burke, 1979). The relative importance of host cell and parasite metabolism in the accumulation of lactate by microsporidan-infected blue crabs can not be determined from this study but will be discussed in connection with isolated parasite carbohydrate metabolism (see Chapter 3).

Microsporidan infection contributes to significant alterations in the biochemical constituents of blue crab host tissues.

Modifications in skeletal muscle protein and carbohydrate metabolism are ultimately reflected in substantial variations in hemolymph composition. Characterization of the blue crab host cell environment during Ameson michaelis infection provides critical background information for metabolic studies concerning isolated microsporidan parasites.

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Chapter 3

Carbohydrate Metabolism of Isolated Microsporidan Parasites

INTRODUCTION

Studies attempting to characterize the metabolic and biochemical properties of obligate intracellular protozoan parasites are subject to several formidable problems: (i) As unicellular organisms, parasitic protozoans necessarily possess biochemical capabilities which are more complex than the specialized activities of metazoan cells. (ii) By viture of their obligate intracellular existence, the metabolic activities of these parasites are intimately associated with those of their host cell environment. (iii) The intricate life cycles of these parasites often involve multiple hosts and/or various specialized tissues within a single host. (iv) Metabolic studies conducted using different developmental stages of the same parasite may produce dissimilar and often conflicting data concerning biochemical characteristics (see Fletcher and Maegraith, 1972; Thompson and Werbel, 1972; Trager, 1974; Rieckmann and Silverman, 1977 for detailed review of this subject).

Any examination of microsporidan energy metabolism is contingent upon suitable techniques for the isolation and extracellular maintenance of the various life cycle stages. Ameson michaelis parasites of the blue crab, Callinectes sapidus, provide a convenient source of cells for such nutritional studies. The particularly resistant nature of the spore wall allows easy isolation of this stage from host muscle cells (Weidner, 1970).

In vitro exposure of purified spores to certain osmotic and ionic shifts results in spore hatching and the isolation of the

expelled sporoplasm stage (Weidner, 1972; 1976). Viable sporoplasms maintained extracellularly for four hours in tissue culture medium supplemented with adenosine triphosphate (ATP), coenzyme A and pyruvic acid retain their ultrastructural integrity (Weidner and Trager, 1973). However, there is no indication of sporoplasm growth or differentiation during the incubation period. This appears to be a natural situation since sporoplasms of several microsporidan species injected into host cells in vitro remain quiescent for some time (up to 24 hr) before displaying obvious evidence of development (Ishihara and Sohi, 1966; Shadduck, 1969; Undeen, 1975).

This initial "static" period may actually be a rather dynamic time when several essential transport and metabolic processes become functionally competent prior to the growth and multiplicative phase of parasite development. As a first step in characterizing the metabolic potential of the early sporoplasm stage, we have examined the carbohydrate activity of sporoplasm populations during brief periods of extracellular maintenance.

MATERIALS AND METHODS

Isolation and Discharge of Spores

Skeletal muscle heavily infected with Ameson michaelis was removed from blue crabs and homogenized in the presence of 0.1 N KOH to dissociate the host tissue component. The resulting suspension was centrifuged (8,000 g; 20 min) and the supernatant material, containing mostly muscle homogenate, was discarded. The crude spore pellet was resuspended in distilled water and centrifuged (1,000 g; 10 min). The centrifugation and wash cycle was continued until a pure white spore pellet was obtained. The purity of the pellet was verified by light microscopy. Isolated spores were stored at 0-4°C in distilled water containing an antibiotic (50 µg/ml gentamycin; Schering Corp.) to suppress bacterial growth.

Spores of Ameson michaelis were primed for hatching with 90-120 min incubation in freshly made Michaelis-Veronal acetate buffer (0.15 M sodium acetate, 0.15 M sodium barbitol in carbon dioxide-free distilled water, pH 10). Following centrifugation (1,000 g; 10 min), the spores discharged when placed in Medium 199 with glutamine, modified Earle's salts and without phenol red indicator, pH 7.2 (Grand Island Biological Co., Cat. No. 320-1157). The resulting sporoplasms were used for the study of their biochemical activity.

Glucose Catabolism

Hatched sporoplasms were incubated in Medium 199 ± 3 mM ATP and 10 mM NaF. At designated intervals (0-180 min), 2 ml aliquots

were withdrawn and the cells were harvested from the incubation medium by centrifugation (10,000 g; 2 min). The glucose concentration of the supernatant medium was quantified colorimetrically by the glucose oxidase-peroxidase method (Raabo and Terkildson, 1960 as modified by Sigma Chemical Co., Kit No. 510). In addition, the accumulation of lactate and pyruvate in the medium was determined. 1.0 ml of the supernatant was immediately transferred to centrifuge tubes containing 2.0 ml cold 8% perchloric acid. The tubes were agitated and returned to an ice bath for 5 min to assure complete precipitation of any proteins present. The mixture was subsequently centrifuged (3,000 g; 10 min) and the lactate and pyruvate concentrations of the protein-free supernatant were quantified by the Sigma method for the ultra-violet determination of lactic and pyruvic acids at 340 nm (Sigma Chemical Co., Kit Nos. 826-UV and 726-UV, respectively).

Glucose Uptake

Primed spores were resuspended for hatching in 10 ml tissue culture media containing 0.5 μCi/ml ^{14}C-D-glucose (uniformly labelled, specific activity 1-5 mCi/mM, New England Nuclear Corp.). At designated intervals (0-120 min), 1 ml aliquots were withdrawn and the sporoplasms were immediately removed from the radioactive medium by centrifugation (10,000 g; 2 min). The supernatant was measured directly for the extracellular disappearance of the ^{14}C-label. The pellet was washed twice with nonradioactive medium, solubilized with Protosol (New England Nuclear Corp.) and assayed for the

intracellular appearance of the ^{14}C-label. Radioactivity was measured using a Beckman liquid scintillation counter. Aquasol-2 and Econofluor (New England Nuclear Corp.) were used as the scintillation cocktails for the supernatant and solubilized cell pellet samples, respectively. Glucose uptake values were obtained by converting the radioactive counts into μmoles glucose taken up/10^{10} sporoplasms. The uptake of glucose by A. michaelis sporoplasms was conducted in Medium 199 or modified Minimum Essential Medium (0.5 mM glucose, minus ATP, leucine and glycine) with or without the addition of 3 mM ATP·tris and in the presence of various inhibitors: 10 mM NaF, 1 mM ouabain and 0.5 mM amiloride.

RESULTS

Following 90-120 min incubation in Michaelis-Veronal acetate buffer (pH 10), Ameson michaelis spores germinated when placed in tissue culture Medium 199 (M 199, pH 7.2). Typically, only 10-20% of the spores hatched -- i.e., ejected their polar filament and expelled an infective spore-cell (sporoplasm or sporoplast) from the distal end of the invasion tube. Spore suspensions were surveyed with a hemocytometer both before and after hatching to obtain an accurate count of the number of sporoplasm cells present. Metabolic data were expressed as a function of sporoplasm number rather than on a dry weight basis; untreated and primed, but unhatched, spores lacked detectable metabolic activity. The decision to refer metabolic measurements to unit number of organisms rather than to unit weight was dictated by the technical difficulties of obtaining reliable dry weight figures. Additionally, since experimental cultures contained both sporoplasms and unhatched spores, which are not easily segregated from one another, their relative contribution to weight measurements can not be accurately determined.

Glucose Catabolism

Ameson michaelis sporoplasms utilized glucose when maintained extracellularly in M 199 supplemented with 3 mM ATP (Table 3-1). The disappearance of exogenous glucose was followed by the appearance of substantial quantities of both lactic and, to a lesser extent, pyruvic acids in the culture medium (Table 3-2 and 3-3). The most rapid rate of glucose consumption occured between 0-30 min of

Table 3-1. Glucose utilization by isolated Ameson michaelis sporoplasms incubated in Medium 199 (M 199) containing 5.5 mM glucose. Data are presented as (cumulative) μmoles glucose utilized/10^{10} sporoplasms. All values are given as the mean of four experiments ± 95% confidence interval.

Time of Incubation (min)	M 199 +3 mM ATP	M 199 - ATP	M 199 + 3 mM ATP +10 mM NaF
30	30.3 ± 4.5	0	0
60	40.0 ± 2.3	0	0
120	46.4 ± 1.2	1.2 ± 0.4	0
180	47.3 ± 1.9	4.7 ± 0.9	0

Table 3-2. Lactate production by isolated Ameson michaelis sporoplasms incubated in Medium 199 (M 199) containing 5.5 mM glucose. Data are presented as (cumulative) µmoles lactate produced/10^{10} sporoplasms. All values are given as the mean of four experiments ± 95% confidence interval.

Time of Incubation (min)	M 199 +3 mM ATP	M 199 - ATP	M 199 + 3 mM ATP +10 mM NaF
30	12.2 ± 3.1	0.6 ± 0.1	0.6 ± 0.4
60	47.9 ± 4.5	1.4 ± 0.9	1.7 ± 0.5
120	62.4 ± 1.7	7.3 ± 0.7	1.6 ± 0.4
180	62.3 ± 0.9	7.3 ± 1.1	1.7 ± 0.5

Table 3-3. Pyruvate production by isolated Ameson michaelis sporoplasms incubated in Medium 199 (M 199) containing 5.5 mM glucose. Data are presented as (cumulative) μmoles pyruvate produced/10^{10} sporoplasms. All values are given as the mean of four experiments ± 95% confidence interval.

Time of Incubation (min)	M 199 +3 mM ATP	M 199 - ATP	M 199 + 3 mM ATP +10 mM NaF
30	23.6 ± 3.0	20.8 ± 2.4	8.5 ± 1.7
60	35.8 ± 4.1	21.6 ± 1.3	7.9 ± 0.9
120	41.4 ± 1.7	20.9 ± 0.5	7.8 ± 0.3
180	41.0 ± 1.0	21.2 ± 0.8	7.5 ± 1.1

sporoplasm incubation (∿30 μmoles glucose utilized/10^{10} sporoplasms) while the highest rate of pyruvate and lactate accumulation took place between the 0-30 min (∿27 μmoles pyruvate produced/10^{10} sporoplasms) and 30-60 min (∿36 μmoles lactate produced/10^{10} sporoplasms) periods, respectively. Subsequently, carbohydrate was consumed at a reduced rate with only minor decreases in medium glucose concentration observed (∿10 μmoles glucose utilized/10^{10} sporoplasms for the 30-60 min incubation period; ∿7 μmoles glucose utilized/10^{10} sporoplasms for the 60-180 min interval). With prolonged incubation, the level of metabolic end-products reached an apparent steady-state condition in which only minor increases in concentrations were noted (∿14 μmoles lactate produced/10^{10} sporoplasms from 60-180 min; ∿5 μmoles pyruvate produced/10^{10} sporoplasms from 60-180 min). During 180 min of sporoplasm incubation, the total production of lactic and pyruvic acids (∿103 μmoles lactate and pyruvate produced/10^{10} sporoplasms = 52 μmoles glucose utilized) slightly exceeded the amount of exogenous glucose utilized (∿ 47 μmoles glucose utilized/10^{10} sporoplasms).

Sporoplasms placed in M 199 without the addition of ATP rapidly (within 15-30 min) disintegrated. In the absence of ATP, medium glucose levels remained virtually unchanged with only a gradual decrease evidenced after 120-180 min of incubation (Table 3-1). When compared to sporoplasms maintained in supplemented media, cells deprived of ATP evolved reduced quantities of pyruvate (∿21 μmoles pyruvate produced/10^{10} sporoplasms after 180 min incubation) and

barely detectable amounts of lactate (∿ 7 μmoles lactate produced/10^{10} sporoplasms after 180 min)(Table 3-2 and 3-3).

The addition of 10 mM NaF, a potent glycolytic inhibitor, to M 199 containing 3 mM ATP eliminated glucose utilization (Table 3-1). Sporoplasms subjected to fluoride treatment did not evolve substantial quantities of either glycolytic end-product surveyed (∿ 1.5-2.0 μmoles lactate produced/10^{10} sporoplasms after 180 min incubation;∿ 7.5-8.0 μmoles pyruvate produced/10^{10} sporoplasms after 180 min) (Table 3-2 and 3-3).

Glucose Uptake

Ameson michaelis sporoplasms maintained in M 199 (5.5 mM glucose) or modified Minimum Essential Medium (0.5 mM glucose) supplemented with 3 mM ATP accumulated glucose supplied in the external medium (Table 3-4). Glucose uptake did not occur appreciably in the absence of an exogenous energy source (i.e., ATP) or in the presence of 10 mM NaF. At low substrate concentrations (0.5 mM glucose), the rate of carbohydrate entry into microsporidan cells (∿ 11 μmoles glucose taken up/10^{10} sporoplasms after 60 min incubation) was more than five times that observed for higher (5.5 mM) glucose concentrations (∿2 μmoles glucoses taken up/10^{10} sporoplasms after 60 min). Following 60 min of sporoplasm incubation, an apparent steady-state condition was achieved in which no further increases in glucose uptake were demonstrated. On the contrary, a gradual decline in the cellular radioactive label accompanied extended maintenance periods (>60 min).

Table 3-4. Uptake of U-^{14}C-D-glucose by isolated Ameson michaelis sporoplasms. Data are presented as moles glucose taken up/10^{10} sporoplasms. All values are given as the mean of three experiments ± 95% confidence interval.

Time of	Low Substrate[a]			High Substrate[b]		
Incubation (min)	+ ATP	- ATP	+ ATP - NaF	+ ATP	- ATP	+ ATP - NaF
30	3.6 ± 0.5	2.1 ± 0.4	1.9 ± 0.3	0.9 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
60	11.2 ± 1.7	1.9 ± 0.2	1.5 ± 0.2	2.2 ± 0.9	0.4 ± 0.2	0
90	10.8 ± 1.1	0.9 ± 0.2	0.4 ± 0.1	2.0 ± 0.7	0	0
120	9.9 ± 1.3	0.5 ± 0.1	0	1.7 ± 0.4	0	0

[a]Modified Minimum Essential Medium containing 0.5 mM glucose

[b]Medium 199 containing 5.5 mM glucose

Glucose uptake by A. michaelis sporoplasms displayed sensitivity toward known inhibitors of sodium transport. At low substrate concentrations, 1 mM ouabain effectively inhibited glucose transport by 75.0 ± 4.5% (a decrease of ∿8.4 μmoles glucose taken up/10^{10} sporoplasms after 60 min incubation), whereas glucose uptake was considerably less affected by ouabain at higher substrate levels (45.2 ± 2.7% inhibition = decrease of ∿1.0 μmoles glucose taken up/ 10^{10} sporoplasms after 60 min). In the presence of 0.5 mM amiloride, glucose entry into cells was also preferentially inhibited at low (52.1 ± 3.5% inhibition = decrease of ∿5.8 moles glucose taken up/ 10^{10} parasites after 60 min incubation rather than high substrate concentrations (34.7 ± 2.1% inhibition = decrease of 0.8 μmoles glucose taken up/10^{10} parasites after 60 min).

DISCUSSION

With few exceptions, parasitic protozoans have been found to utilize or even require soluble carbohydrates (von Brand, 1973). Therefore, a carbohydrate source, usually in the form of glucose, is frequently incorporated as a major constituent of media used in their extracellular cultivation. It is evident, however, that a definitive statement concerning carbohydrate utilization by intracellular parasites can be made only if the parasites are completely isolated from host cells and are maintained extracellularly as pure populations. Caution must be exercised, nevertheless, in the interpretation of data derived from the in vitro manipulation of intracellular parasites. Since culture conditions cannot faithfully approximate the host cell environment, it is quite possible that biochemical potentialities are being studied rather than metabolic realities as they occur in vivo (von Brand, 1973).

In all cells, the catabolism of glucose leads to the release of chemical energy which is coupled to the synthesis of high-energy compounds {i.e., adenosine triphosphate (ATP)} needed to drive the cell's anabolic processes. Maximum energy yield is achieved in most cells by the complete oxidation of glucose to carbon dioxide and water with the assistance of the mitochondrion and its associated tricarboxylic acid (TCA) cycle and oxidative phosphorylation. However, mitochondria appear to be absent from all stages of microsporidan development (Weidner, 1970). Therefore, it appears likely that glycolysis is a major energy-yielding catabolic pathway

in these parasites.

Ameson michaelis sporoplasms readily utilize glucose when maintained extracellularly in tissue culture Medium 199 (M 199) supplemented with 3 mM ATP. A decline in exogenous glucose is followed by the appearance of substantial quantities of both lactic and pyruvic acids in the culture medium. The most rapid rate of glucose utilization occurs between 0-30 min of sporoplasm incubation while the highest rate of pyruvate and lactate accumulation takes place between the 0-30 and 30-60 min periods, respectively. Subsequently, glucose is consumed at a reduced rate and the level of metabolic end-products reaches an apparent steady-state condition in which no further increases in concentration are observed. A similar situation has been reported for the carbohydrate metabolism of freed erythrocytic forms of Plasmodium berghei (Bowman et al., 1960). The total production of lactic and pyruvic acids ($\sim$103 µmoles lactate and pyruvate produced/10^{10} parasites = 52 µmoles glucose utilized) slightly exceeds the amount of exogenous glucose utilized ($\sim$ 47 µmoles utilized/10^{10} parasites). Therefore, it appears quite probable that sporoplasms are exploiting some endogenous carbohydrate source. Spores of Nosema apis possess a functional trehalase system (Vandermeer and Gochnauer, 1971). Trehalase may also be present in Ameson michaelis parasites (Weidner, unpublished data). The activity of such an enzyme in microsporidan sporoplasms may furnish a substantial amount of the glucose required during early parasite growth.

Sporoplasms placed in M 199 without the addition of ATP rapidly

(within 15-30 min) begin to disintegrate. This disintegration includes the dissociation of the outer cell membranes as well as the collapse of the internal structure (Weidner and Trager, 1973). In the absence of ATP, the medium glucose concentration remains virtually unchanged indicating that an exogenous energy source may be necessary for glucose utilization by microsporidan parasites. When compared to sporoplasms maintained in supplemented media, cells deprived of ATP evolve reduced quantities of pyruvate and barely detectable amounts of lactate. A portion of the pyruvate may be attributable to the leakage of glycolytic enzymes from deteriorating cells. However, since sporoplasms do not appear to significantly consume exogenous glucose in the absence of ATP, the production of pyruvate is more likely due to the utilization of some endogenous carbohydrate source. The accumulation of pyruvate in the culture medium may result from the leakage of this metabolite through the disorganized membranes of cells maintained without ATP, prior to its further metabolism to lactate.

The presence of 10 mM NaF to M 199 containing 3 mM ATP virtually eliminates glucose consumption. NaF represents a potent inhibitor of the glycolytic enolase reaction. With the addition of fluoride, Mg^{2+} (a divalent cation for which enolase has an absolute requirement) is included in an inhibitory species, magnesium fluorophosphate, which complex with the enzyme thereby preventing substrate binding (Lehninger, 1975; White et al., 1978). Sporoplasms exposed to NaF do not consume exogenous glucose nor do they evolve substantial quantities of glycolytic end-products.

Glycolysis appears to be a major pathway of glucose metabolism in microsporidan parasites. Glucose consumption leads to the production of significant quantities of lactate and, to a lesser extent, pyruvate. The relative importance of these metabolites as the in vivo end-products of glycolysis remains unclear. In bloodstream forms of trypanosomes, aerobic and anaerobic glucose fermentation results in the generation of large amounts of pyruvate and only trace amounts of lactate (Grant and Fulton, 1975; Bowman et al., 1970). In malaria organisms, however, the opposite trend has been recorded and lactate is the principle end-product of parasite glycolysis (Wendel, 1943; Silverman et al., 1944; Speck et al., 1946; Fulton and Spooner, 1956; Bowman et al., 1960; Sherman et al., 1969).

The rates of glucose utilization and lactate or pyruvate production by parasitic protozoans are typically quite variable and display parasitic strain, environmental and life cycle stage differences (von Brand, 1973). In addition, it is often rather difficult to compare precise numbers from individual studies because sugar consumption usually is referred to unit number or volume of organisms rather than to unit weight because of the technical difficulties of obtaining reliable dry weight figures (von Brand, 1973). However, values obtained for glucose utilization and lactate production by A. michaelis sporoplasms correspond well to data from several Plasmodium species (Speck et al.,1946; Bowman et al., 1960) and Toxoplasma gondii (Fulton and Spooner, 1960).

The production of lactate by isolated microsporidan parasites

may represent a significant contribution to the accumulation of this metabolite in the thoracic skeletal muscle of infected blue crabs. In light of information supplied by Ameson michaelis sporoplasms it seems reasonable to assume that sporoblasts of muscle are also capable of producing substantial quantities of lactate. Additionally, in its stressed condition, host muscle tissue is likely to be a rather anaerobic environment. The absence of oxygen would further enhance the production of lactate by both host cells and microsporidan parasites.

If we assume lactate to be the major end-product of microsporidan fermentation, the presence of substantial amounts of pyruvate may be rationalized through one or more of the following explanations: (i) The leakage of pyruvic acid to the surrounding medium may be an artifact of unfavorable in vitro conditions. (ii) Since microsporidans lack mitochondria, pyruvate may indeed be passed to the host cell in vivo for its compelte oxidation to carbon dioxide and water. (iii) Despite the absence of mitochondria, microsporidans may still possess functional portions of the tricarboxylic acid cycle. However, because the TCA cycle would be incomplete in the parasite, host cell machinery may be required for the cooperative handling of pyruvate. This last alternative actually has considerable factual basis since some of the enzymes of the TCA cycle (i.e., aconitase, fumarase and malate dehydrogenase) are also found in the extramitochondrial cytoplasm (Lehninger, 1975). In addition, microsporidan parasites are frequently found suspiciously near host cell mitochondria

during their growth phase of development (Weidner and Trager, 1973).

Observations concerning the relative importance of glycolysis and the TCA cycle in the carbohydrate metabolism of avian malaria parasites may also be pertinent to the situation in microsporidans. Malaria organisms have mitochondria and appear to possess a completely functional TCA cycle. However, several studies have indicated that glycolysis is the major pathway of glucose catabolism in Plasmodium (Moulder, 1962; Sherman et al., 1970). Malaria parasites maintained extracellularly following isolation from host erythrocytes lose key intermediates of the TCA cycle through diffusion (see Seed and Manwell, 1977). If this condition occurs in vivo as well, it may explain the inefficiency of the TCA cycle in malaria parasites and the possibility of host cell intervention in the metabolism of parasitic glycolytic intermediates (Moulder, 1962).

Despite numerous investigations detailing carbohydrate metabolism in parasitic protozoans, relatively few studies have addressed the question of glucose transport into these cells. The use of appropriate analogs to inhibit the uptake and utilization of glucose by bloodstream forms of trypanosomes suggested the probable occurrence of a mediated transport mechanism (Seed et al., 1965). Additionally, entrance of carbohydrates into cells at low and high substrate concentrations may proceed by dissimilar mechanisms (Min, 1965; 1966). In the insect trypanosomid, Crithidia luciliae, data concerning penetration kinetics, specificity, Q_{10} and effects of metabolic inhibitors indicate that carbohydrate uptake occurs by an active transport mechanism at

low (0.5 mM0 external substrate concentration, whereas facilitated diffusion predominates at higher (20 mM) concentrations (Min, 1965; 1966).

Ameson michaelis sporoplasms are capable of accumulating glucose from the external medium. The rate of glucose uptake at low (0.5 mM) substrate concentrations (∿11 μmoles/10^{10} sporoplasms/hr) is greater than that observed for higher (5.5 mM) concentrations (∿2 μmoles/10^{10} sporoplasms/hr). Following a 60 min incubation period, an apparent steady-state condition is achieved in which no fruther increases in glucose uptake are observed. In fact, with prolonged incubation (>60 min), a gradual decline in the cellular radioactive label is observed. This result may be attributable, in part, to the use of a readily metabolizable sugar since the decrease in cell activity roughly coincides with the appearance of lactate and pyruvate in the culture medium.

Radioactive tracer measurements of glucose uptake by microsporidan cells is considerably lower than might be expected from enzymatic determinations of glucose disappearance form the culture medium. This observation may be a significant consequence of the dilution of labelled glucose by a larger unlabelled substrate pool, a situation which is exacerbated by the low specific activity of the labelled material (i.e., decreased sensitivity of the assay method). In addition, glucose transport values may be artificially lowered by the exit of ^{14}C-labeleled end-products (i.e., pyruvate and lactate) from rapidly metabolizing cells.

In many cell types, the transport of certain sugars is coupled to the continual progress of sodium ion movement (Na^+-gradient dependent sugar transport) (Crane, 1962; Crane et al., 1978). According to a current model, cation and substrate are thought to join with a specific protein carrier to establish a ternary complex which co-transports both species across the membrane barrier (Crane et al.,1978). Therefore,the uptake of Na^+-dependent sugars (i.e., glucose) by cells is determined by the presence and concentration of sodium ions. Additionally, the amount of sugar accumulation is a function of energy available in transmembrane ion gradient generated by the Na^+ pump (Crane, 1977).

The interaction of sodium ion and sugar transport may be discerned by effectively eliminating one process and demonstrating the other to be inhibited as well. For example, phlorizin, an inhibitor of sugar transport, has an action on sodium movement in the presence of Na^+-dependent sugars, but not in their absence (Crane, 1962; Crane et al., 1978). Conversely, specific poisons of active sodium movement, such as ouabain or amiloride, inhibit the movement of Na^+-dependent sugars (Clausen, 1966).

Glucose uptake by Ameson michaelis sporoplasms displays sensitivity toward known inhibitors of sodium transport. The energy-dependent extrusion of Na^+ from cells is very specifically inhibited by the cardiac glycoside, ouabain. At low substrate concentrations (0.5 mM), 1 mM ouabain effectively inhibits (by ~75%) glucose transport, whereas glucose uptake is considerably less affected

by ouabain (~45% inhibition) at higher substrate levels (5.5 mM). These data indicate that glucose uptake may occur by dissimilar mechanisms at low and high substrate concentrations. The usefulness of ouabain as a potent inhibitor of Na^+ efflux may be impaired by the high concentration of external K^+ ions. Potassium has been shown to impede the binding of cardiac glycosides to ion pumping sites (Baker and Willis, 1970). In the presence of 0.5 mM amiloride, the transport of glucose into cells is also preferentially inhibited at low rather than high substrate concentrations. However, inhibition by amiloride is not as dramatic as evidenced with ouabain treatment. The effectiveness of amiloride has probably been compromised to some extent by the high sodium concentration of the medium.

Sodium fluoride, an inhibitor of glycolysis, is also an effective inhibitor of glucose entry into microsporidan cells. The exact mechanism for this action remains unclear however, fluoride may significantly alter cellular Na^+ movement. 30 mM NaF virtually eliminates sodium efflux from the bacterium, Escherichia coli (Schultz and Solomon, 1961). Additionally, fluoride may affect glucose transport indirectly by abolishing the primary energy-yielding pathway in these parasites thereby eliminating cellular ATP pools.

Our data suggest that glucose transport in microsporidan cells is mediated through a Na^+-dependent process. The relative importance of such a mechanism appears to differ at low and high substrate concentrations. Min (1965; 1966) suggested that carbohydrate transport in Crithidia luciliae proceeds almost exclusively by either

active transport or facilitated diffusion at low and high substrate levels, respectively. In Ameson michaelis, however, such a sharp distinction cannot be made. Rather the preeminence of Na^+-dependent versus Na^+-independent sugar transport varies with the glucose level of the culture medium.

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EXAMINATION AND THESIS REPORT

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Approved:

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18 April, 1980